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Diabetes, lower extremity amputation, loss of protective sensation, and NOS1AP in the CRIC study

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Abstract

Lower extremity amputation (LEA) is a life-altering complication of diabetes. The goal of our study was to investigate the possibility that genetic variation in neuronal nitric oxide synthase associated protein *(NOS1AP)* is associated with LEA and diabetic peripheral neuropathy (DPN). Our work used data from the Chronic Renal Insufficiency Cohort (CRIC) study. CRIC is a multicenter investigation undertaken to pursue the relationship between chronic renal insufficiency and cardiovascular disease. We evaluated 3,040 CRIC study subjects, 1,490 individuals were African-Americans and 1,550 were whites. LEA occurred in 162 (5.3%) subjects, 93 (6.2%) of African-Americans and 69 (4.4%) of whites. In whites, *NOS1AP* SNP **rs1963645** was most strongly associated with LEA (1.73 (1.23, 2.44)). In African-Americans three *NOS1AP* SNPs were associated with LEA: **rs6659759** (1.65 (1.21, 2.24)); **rs16849113** (1.58 (1.16, 2.14)); **rs880296** (1.54 (1.14, 2.10)). We tested a subset of 100 CRIC participants for DPN using Simmes-Weinstein filaments. DPN in those with diabetes was associated with **rs1963645** (16.97 (2.38, 120.97)) in whites and **rs16849113** and **rs6659759** (3.62 (1.11, 11.83) and 3.02 (0.82, 11.12) respectively) in African-Americans. In conclusion, this is one of the first studies to show that NOS1AP gene variants are associated with DPN and LEA.

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INTRODUCTION

Lower extremity amputation (LEA) is an expensive and life altering complication of diabetes. In 2009, among those enrolled in Medicare, LEA was associated with yearly costs between $30,000 and $60,000 and was associated with a 20% annual increased risk of mortality (1,2). Rates of LEA have decreased over the past 10 years but the risk of amputation varies substantially by race/ethnic group, sex, and geographic location(2). About 34% of those who have an amputation will have a second more extensive one within 16 weeks of their initial amputation(3).

Individuals with diabetes develop LEA for many reasons. Most are due to the development of a foot ulcer and the failure of that foot ulcer to heal. Studies have shown that about 90% of individuals with LEA had a history of a foot ulcer and the foot ulcer is most often associated with peripheral vascular disease (PVD) and/or diabetic peripheral neuropathy (DPN) of the lower extremity (4,5). DPN of the foot leads to loss of protective sensation (LOPS) doubling the likelihood of developing a foot ulcer and tripling the risk of a lower extremity amputation (LEA)(2,5-7). LOPS/DPN is also associated with the loss of ankle reflex and the loss of musculature of the leg and foot, thereby resulting in a foot strike that causes the foot to bear excessive and repeated trauma while walking. The mere act of walking on an insensate foot can result in skin damage leading to a wound. Studies in mice have also demonstrated that loss, or alterations, of peripheral innervation profoundly affects the ability of the animal to repair cutaneous wounds(8). Beyond clinical examinations and assessments of vascular flow, there are no objective markers with which to assess the risk for a LEA. Consequently, identification of genetic variations associated with increased risks would have great value in clinical management of those with diabetes.

An interesting candidate gene is neuronal nitric oxide synthase associated protein, *NOS1AP* (1q23.3). *NOS1AP* encodes a cytosolic protein that binds to neuronal nitric oxide synthase (gene-NOS1 or protein-nNOS) via an N-terminal phosphotyrosine binding (PDZ) domain (9). *NOS1AP*, stabilizes nNOS potentiating its subcellular influence(9). As a result *NOS1AP* enhances the activity of nNOS to activate and bind to G-proteins(9). Interactions between nNOS and *NOS1AP* and *NOS1AP* direct interactions may explain the findings that genetic variation of *NOS1AP* have been associated with cardiac arrhythmia, schizophrenia as well as inconsistently with diabetes(10-15).

The goal of our study was to investigate the possibility that genetic variation in *NOS1AP* is associated with LEA, and to evaluate whether this association could be related to LOPS, which appears to be part of the causal pathway to LEA(5). Our work used data from the Chronic Renal Insufficiency Cohort (CRIC) study, an NIH-sponsored cohort study of individuals with chronic kidney disease (CKD)(16-18). The CRIC study has collected data on lower extremity amputation during yearly examinations.

METHODS

POPULATION

CRIC is a multicenter investigation undertaken to pursue the relationship between chronic renal insufficiency and cardiovascular disease (19,20). The CRIC clinical research centers are located at the University of Pennsylvania, Johns Hopkins University/University of Maryland, Case Western Reserve University, University of Michigan, University of Illinois-Chicago, Tulane University Health Science Center, and Kaiser Permanente of Northern California/University of California at San Francisco. All subjects are examined at their local CRIC site each year. Briefly, the yearly visit includes history, physical, and laboratory evaluations focused on factors that might contribute to or explain rate of progressive loss of

kidney function in CKD and its relation to the progression of chronic renal insufficiency and atherosclerotic vascular disease. These evaluations include information on lower extremity amputation, lower extremity peripheral vascular disease (PVD), diabetes, initial hemoglobin A1c (HgbA1c), gender, race/ethnicity, age, body mass index, estimated glomerular filtration rate (eGFR), and ankle brachial index. All subjects enrolled in CRIC had decreased kidney function as defined by the Modification of Diet in Renal Disease equation(21). About half of the CRIC participants have diabetes. We received the dataset from CRIC August 2011.

Additional evaluations—A routine diabetic foot examination is not conducted on CRIC subjects. Hence, in order to test our hypothesis we conducted a standard evaluation of lower extremity sensation using single-use 10 gram 5.07 level tactile monofilament evaluator (Semmes-Weinstein filaments) on 100 consecutive CRIC subjects seen at the University of Pennsylvania. This study was approved by the CRIC Steering Committee and the University of Pennsylvania's Institutional Review Board. At the study visit, subjects consented to participate in the lower extremity sensation exam. All subjects who were approached agreed to participate. All subjects were evaluated in a supine position. The coordinator conducting the filament testing was blind to all phenotypic and genetic information. Individuals with a previous history of amputation were excluded from evaluation. With their eyes closed, subjects were asked to respond by saying “yes” if they felt the monofilament on either foot. The investigator tested the plantar surface of the 1st, 3rd and 5th metatarsal head of each foot and the plantar surface of the great and 4th toe on both the right and left foot. Monofilaments are constructed to buckle when force is applied; loss of the ability to detect this pressure at one or more sites on either foot was recorded as LOPS.

Genetic Variation—Subjects who agreed to participate in the CRIC study were separately consented by CRIC to participate in studies of their genetic variation. Available genotype information of CRIC participants was derived from the ITMAT-Broad-CARe array (IBC) chip, which spans approximately 50,000 single nucleotide polymorphisms (SNP) within selected genes and is described in more detail elsewhere(17). From the IBC chip, we *a priori* selected SNP markers for evaluation for their potential association with wound repair and oxidative stress. Specifically, *NOS1AP* was selected because of its potential association with neuronal growth, oxidative stress, and cardiovascular disease(10,11). In addition, because neuropathy and foot ulcers are somewhat common among those with diabetes, we *a priori* selected only SNP markers with a minor allelic frequency of greater than 30%. Based on these criteria, we evaluated a total of 44 *NOS1AP* SNPs for the white cohort and 51 *NOS1AP* SNPs for the African American cohort from the IBC chip(17).

Genetically inferred race/ethnicity previously was determined by CRIC investigators using principal components analyses as described in Price *et al* (22). Thus, we received *NOS1AP* genotypes within the following four categories: non-Hispanic white, non-Hispanic black, Hispanic, and Asian/other. Due to sample size concerns, we only studied non-Hispanic white (aka whites) and non-Hispanic black (aka African-Americans). All CRIC data were evaluated separately based on these groupings.

Analysis—We estimated allelic and genotypic frequencies for each SNP. Chi-squared tests were conducted to determine whether the allelic distributions at each locus were in Hardy-Weinberg equilibrium (HWE). Our primary goal was to estimate the association of common *NOS1AP* SNPs first with respect to LEA and then to use these data to evaluate the potential for an association with respect to LOPS in the 100 subjects tested from the UPENN CRIC cohort subset. Tests of association between SNPs and binary outcomes were conducted using chi-squared tests and logistic regression (assuming an additive genetic model) using PLINK and STATA (version 12.1, College Station, TX), as appropriate. Because this was an exploratory analysis, we used a cut-off of $p \leq 0.05$ and the criteria listed above for

determining which SNPs we would evaluate with respect to LOPS. False-discovery rate corrected P-values (P_{corr}) are presented for the primary associations of LEA among those with diabetes (23). We did not correct the LOPS P-values for multiple comparisons because this was an exploratory analysis intended to identify specific *NOS1AP* SNPs for future investigation.

To investigate haplotype blocks within the gene of interest, LD heat maps were generated using Haploview(24). The EM algorithm was used to estimate haplotype frequencies and haplotype association tests were conducted using Schaid's generalized linear models approach using the program haplo.stats in R version 2.13. Haplotype-specific tests, to test the association between each haplotype and disease status, were conducted using haplo.glm(25). We created only two custom haplotype blocks, one each for those classified as whites and African-Americans

RESULTS

Our CRIC study sample consisted of 3,040 study subjects, of which1,490 individuals were African-American and 1,550 individuals were white (Table 1). Overall, LEA occurred in162 (5.3%) of which 93 (6.2%) individuals were African-American and 69 (4.4%) individuals were white. Diabetes was diagnosed in 46% of our CRIC sample. Furthermore, 47.7% were female, 7.4% had a clinical history of PVD, and 13.0% had an ankle brachial index of less than 0.90. The mean values for age at enrollment, BMI, and eGFR were 58.6 years (sd 10.8), 32.3 KG/m^2 (sd 7.9), and 43.7 ml/min/1.73m^2 (sd 13.3), respectively. More detailed information is presented in Table 1. Participants with diabetes (Odds Ratio: 8.18 (95% Confidence Interval: 5.19, 12.90)), PVD (9.34 (6.50,13.42)), lower ABI (0.32 (0.14,0.74)), and diminished eGFR (0.97 (0.96,0.98)) were more likely to have LEA.

In whites, **rs1963645** most strongly associated with LEA (see Tables 2 and 3). Carriers of the **rs1963645** G-allele were nearly two times [1.73 (1.23, 2.44) p=0.002, p_{corr}=0.061 and in just those with diabetes; 1.90 (1.29, 2.81), p=0.001] more likely to have an LEA. This variant is located at 1:162333990, is an upstream variant (A>G substitution), has an allelic frequency of 38.5%, and was in HWE. We also conducted an adjusted analysis using the following potential confounders: age, diabetes, sex, BMI, HgbA1c, PVD and eGFR. The results were similar to that of the unadjusted analysis [1.80 (1.21, 2.67)]. In those who did not have diabetes, **rs1963645** was not found to be associated with LEA [1.13 (0.48, 2.64)]; however this was based on only 11 LEA outcomes Further, **rs1963645** was not found to be significantly associated with diabetes [OR: 1.04 (0.90, 1.21)].

In African-Americans with diabetes, three SNPs were associated with LEA (see Tables 2 and 3). Carriers of the **rs6659759** C-allele were about 65 percent more likely to have a lower extremity amputation in the full African-American cohort [1.65 (1.21, 2.24), p=0.001 and p_{corr}=0.074] and in just those with diabetes [1.70 (1.21, 2.39), p=0.002]. This variant was located at 1:162037609, had an allelic frequency of 35.4%, was an upstream variant (T>C substitution), and was in HWE. The adjusted results [1.64 (1.13, 2.39)] were similar to that of the unadjusted analysis. The SNP was not associated of having diabetes [0.96 (0.82, 1.12)]. African-American carriers of the **rs16849113** T-allele were about 50 percent more likely to have a lower extremity amputation [1.58 (1.16, 2.14), p=0.003 and p_{corr}=0.093] as well as in just those with diabetes [1.59 (1.14, 2.22), p=0.006]. This variant was located at 1:162040878, had an allelic frequency of 44.5%, was intronic (C>T substitution), and was in HWE. The adjusted results [1.60 (1.20, 2.32)] were similar to that of the unadjusted analysis. The SNP was not associated with diabetes [1.01 (0.88, 1.18)].Carriers of the **rs880296** G-allele were about 50 percent more likely to have a lower extremity amputation [1.54 (1.14, 2.10), p=0.005 and p_{corr} =0.11] and in those with diabetes [1.49 (1.07, 2.09),

p=0.02]. This variant was located at 1:162128446, had an allelic frequency of 35.5%, was an intronic variant (C>G substitution), and was in HWE. The results were similar to that of the unadjusted analysis [1.77 (1.23, 2.57)]. It was not predictive of having diabetes [1.08 (0.93, 1.26)]. Although there were very few LEA outcomes in those without diabetes (N=11), these variants were not predictive of LEA in those who did not have diabetes.

In a haplotype analysis of whites, a haplotype block of 5 SNPs including the SNP most significant in single SNP analysis was inferred. This block included rs7521206 (C>G), rs17428733 (T>A), rs1963645 (A>G), rs905720 (C>T), and rs1964052 (C>T). The haplotype GTACC was significantly associated with LEA in the unadjusted analysis as well as in the analysis adjusted for covariates. It had a frequency of 35.8% overall, and was noted in 27.6% of those with LEA and 36.1% of those without LEA, suggesting a protective effect (OR (crude) = 0.56, 95% CI [0.37, 0.84], p=0.005; OR (adjusted) = 0.54, 95% CI [0.35, 0.83], p=0.006). In African-Americans, a haplotype block consisting of 5 SNPs, which included two of the three SNPs that were associated with LEA in the single SNP analysis, was constructed. This block comprised of the following 5 SNPs: rs6659759 (T>C), rs10918602 (T>C), rs10800279 (T>C), rs1123217(C>G), and rs16849113 (C>T). Two haplotypes, CTCCT and CTTCT were individually associated with LEA in the unadjusted analysis as well as in the analysis adjusted for covariates. The haplotype CTCCT had a frequency of 16.1% overall and was noted in 22.2% of those with LEA and 15.7% of those without LEA, suggesting a deleterious effect (OR (crude) = 2.0, 95% CI [1.3, 3.2], p=0.004; OR (adjusted) = 2.3, 95% CI [1.3, 4.0], p=0.005). The haplotype CTTCT had a frequency of 8.3% overall and was noted in 12.1% of those with LEA and 8.1% of those without LEA, again suggesting a deleterious effect (OR (crude) = 2.1, 95% CI [1.2, 3.6], p=0.01; OR (adjusted) = 2.2, 95% CI [1.1, 4.4], p=0.02).

In order to better understand the etiology of the association between LEA and these SNPs, LOPS using Simmes-Weinstein filaments was determined in 100 consecutive subjects (47 whites, 50 African-Americans, and 3 others) during their yearly examination at the UPENN CRIC study site (see Tables 4 and 5). The data from three subjects who were not white or African-American were not analyzed and are not presented here because of the small sample size Among whites, 16 (34%) were not able to perceive protective sensation at one or more sites on their feet. Of those with diabetes 46% (12 of 26 subjects) had LOPS. LOPS was also noted in 19% (4 of 21) of those who did not have diabetes (i.e. those without diabetes but with CKD). Among diabetics, those with the variant allele of **rs1963645** were almost seventeen times more likely to have LOPS [16.97 (2.38, 120.97)] as compared to those without the allele. Among African-Americans, 26 (52%) were not able to perceive sensation at one or more sites. Of those with diabetes, 60% (18 of 30 subjects) had LOPS. LOPS was also noted in 40% (8 of 20 subjects) of those who did not have diabetes. Among diabetics, subjects with the variant alleles of **rs16849113** and **rs6659759** were nearly 4 and 3 times more likely to have LOPS (3.62 [1.11, 11.83] and 3.02 [0.82, 11.12]) respectively; **rs880296** was not associated with LOPS (1.56 [0.51, 4.77]).

Discussion

We hypothesized that genetic variation of *NOS1AP* gene in association with diabetes was associated with DPN and thus with LEA. We observed four genetic variants of *NOS1AP*, **rs1963645** in the white cohort and **rs16849113, rs6659759,** and **rs880296** in the African-American with diabetes cohort that were associated with at least a 50% increased risk of LEA. With respect to LOPS, a marker for diabetic peripheral neuropathy, we observed that **rs1963645** and **rs16849113** increased the risk of LOPS nearly 17 and 4 times respectively. To the best of our knowledge, this is the first study to observe a genetic association with DPN in those with diabetes.

Diabetic neuropathy refers to several syndromes that include diffuse (e.g., distal symmetric sensorimotor polyneuropathy (e.g., DPN and LOPS) and diabetic autonomic neuropathy) and focal (e.g., mononeuropathy, plexopathy, radiculopathy, etc.) neuropathies (6,26-28). These neuropathies can result in loss of sensation, pain syndromes, cardiovascular disease, gastrointestinal disease, genitourinary disease, decreased mobility, erectile dysfunction, poor wound healing, and limb amputation (6,26-28). Diabetic peripheral neuropathy is one of the most common long-term consequences of diabetes and can lead to the loss of protective sensation of the extremities (6,27,28). Only 20 to 50% of those with diabetes will have DPN and develop LOPS(6). Individuals with diabetes and DPN leading to LOPS are more than twice as likely to develop a foot ulcer and three times more likely to have an LEA(5,29,30). In fact some have suggested that DPN/LOPS is in the causal pathway to the development of foot ulcers and ultimately LEA(5). It has been hypothesized that DPN occurs as a consequence of persistent hyperglycemia or due to glyclation endproduct accumulation resulting in oxidative stress producing nerve injury (31,32). It is, however, not clear whether intensive treatment resulting in tight glycemic control prevents DPN and LOPS (6,7,33). Finally, it is important to realize that not *all* individuals with diabetes develop lower extremity wounds, LEA, DPN, or PVD. Our findings might help to explain these observations in that only those with a genetic predisposition (*i.e.* those with the above identified *NOS1AP* genetic variation) in the setting of hyperglycemia or, perhaps, some other metabolic stressor will develop neuropathy.

Ultimately, the risk of LEA in those with diabetes is dependent on wound factors, comorbidities like chronic kidney disease and peripheral vascular disease, as well as gender, race/ethnicity and many other personal, social, and demographic factors(34-39). In our study we observed that genetic variation in *NOS1AP* is associated with LEA. More specifically, r**s1964052** and **rs16849113** are common SNPS associated with LEA but even more strongly associated with DPN as defined by LOPS in whites and African-Americans respectively. Based on haplotype analysis these SNPs represent potentially important areas of future genetic exploration. Because these variants are common in the HapMap studied populations and CRIC, we speculate (though we lack empirical evidence) that these *NOS1AP* variants do not result in an absence of a nNOS effect but a diminished effect that synergistically interacts with other metabolic impairments like diabetes or CKD to increase the risk of neuropathy ultimately leading to LEA.

It is interesting to note that in our study of 100 UPENN subjects with CKD, LOPS was noted in 19% of non-diabetic whites and 40% of non-diabetic African-Americans. Our study was not designed to precisely evaluate this finding. LOPS is not commonly studied in those with CKD. However, foot problems, foot ulcers, and LEA are very common among those with end-stage renal disease (40). Interestingly, a previous study reported that about 36% of those with CKD who did not have diabetes had LOPS(41). Future studies should more carefully examine this potentially important clinical issue.

There are limitations to our study. First, in order to rule out spurious associations, studies of genetic variation need to be confirmed in other populations. Our study is exploratory and was not designed to find the “causal” genetic variant. However, it is important to remember that the association with our final outcome (LOPS) was based on variants pre-screened for association with LEA. Only four SNPs of interest were evaluated independently with respect to LOPS. These analytic decisions were all made *a priori*. As a result, it is unlikely that our findings were due to false discovery. In addition, the sample size for the LOPS evaluation was small. Second, it is possible that our LOPS testing was not definitive. Many different techniques are used to determine if a patient has LOPS. Simmes-Weinstein filaments are commonly used but more specific and sensitive tests are available and we encourage their use in future studies(42). However, in our study, the investigator conducting the testing was

unaware of the genetic status of the study subject. Further, Simmes-Weinstein filaments, which may not be the gold standard test for DPN, it is certainly an adequate test and commonly used as a neuropathy test in clinical practice. Importantly, in our study the investigator conducting testing was unaware of the genetic status of the study subject. Third, a few large GWAS studies have linked variation in *NOS1AP* to type 2 diabetes but these findings have not been consistent(43). At least one study had concluded that the association at best was weak(44). In our study we did not find an association between our candidate SNPs and diabetes. A potential reason for the previously described weak association might be that *NOS1AP* is not associated with diabetes *per se* but with a complication of diabetes (i.e., DPN/LOPS) that increased the likelihood for a subject to be in a cohort for genetic testing. While many studies of genetic variation and diabetes have been completed, we were not able to find any that specifically evaluated *NOS1AP* genetic variation and neuropathy. Finally, the clinical reasons for LEA do vary by healthcare provider and patient. The decision to amputate likely includes the failure of a wound to heal as well as other important health concerns. We cannot precisely determine which part of this process is influenced by NOS1AP, but since genetic variants in *NOS1AP* are associated with both LEA and LOPS while being unknown to both the patient and the healthcare provider, it is unlikely to be associated with practice variation or patient acceptance of therapy.

In conclusion, we present the first potential association between genetic variation and DPN. We also show that *NOS1AP,* a gene that has been weakly associated with type 2 diabetes, may be associated with LEA in those with diabetes. It is possible that in previous studies, the association with diabetes was biased due to an unintended oversampling of those with diabetic complications. Though not definitive, our results are the first step in furthering our understanding of genetic variation, diabetes and lower limb peripheral neuorpathy. Larger studies are needed to replicate these findings. Future studies should also include isolation of the mutant protein(s), confirmation of the function of the *NOS1AP* protein both mutant and wild type, and then ultimately the development of agents that can alter the activity of *NOS1AP* thereby changing the clinical course of DPN.

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Abbreviations

A	Adenine
ABI	Ankle brachial index
BMI	Body mass index
C	Cytosine
CKP	Chronic kidney disease
CRIC	Chronic Renal Insufficiency Cohort
DPN	Diabetic peripheral neuropathy
eGFR	Estimated glomerular filtration rate
G	Guanine
HgbA1c	Hemoglobin A1c

HWE	Hardy-Weinberg equilibrium
IBC	ITMAT-Broad-CARe array chip
LEA	Lower extremity amputation
LOPS	Loss of protective sensation
MAF	Minor allelic frequency
nNOS	neuronal nitric oxide synthase
NOS1AP	neuronal nitric oxide synthase associated protein
OR	odds ratio
$\mathbf{P_{corr}}$	Corrected P-values
PVD	Peripheral vascular disease
SNP	Single nucleotide polymorphisms
sd	Standard deviation
T	Thymine

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Table 1

Baseline characteristics (raw number and percentage) for those with and without a lower extremity amputation (LEA).

	White N=1550		African-American N=1490	
	No LEA	**LEA**	**No LEA**	**LEA**
Subcohort N(%)	1481 (95.6)	69 (4.4)	1397 (93.8)	93 (6.2)
Women N(%)	603 (96.0)	25 (4.0)	731 (95.9)	31 (4.1)^∧
Age by category N (%)				
<40	106 (98.2)	2 (1.8)	97 (94.1)	6 (5.8)
>=40 - <50	169 (98.8)	2 (1.2)	170 (95.0)	9 (5.0)
>=50 - <60	376 (93.3)	27 (6.7)	424 (92.6)	34 (7.4)
>=60 - <70	578 (95.8)	25 (4.2)	516 (93.6)	35 (6.4)
>=70	252 (95.1)	13 (4.9)*	190 (95.5)	9 (4.5)
Diabetes N (%)	569 (90.8)	58 (9.2)^∧	690(89.4)	82 (10.6)^∧
eGFR ml/min/1.73m^2				
mean (sd)	44.0 (12.8)	39.2 (10.4)#	43.9 (13.9)	39.5 (13.9)#
eGFR > 60 N(%)	150 (98.7)	2 (1.3)	177 (95.7)	8 (4.3)
eGFR 30 – 60	1086 (95.4)	52 (4.6)	977 (94.4)	58 (5.6)
eGFR < 30	245 (94.2)	15 (5.8)*	243 (90.0)	27 (10.0)
ABI mean (sd)	1.11 (0.2)	1.11 (0.4)	1.06 (0.2)	0.99 (0.2)*
ABI <0.90 N(%)	139 (90.8)	14 (9.2)^∧	210(90.51)	22 (9.4)*
Diagnosis of PVD N(%)	71 (71.0)	29 (29.0)^∧	83 (75.5)	27 (24.5)^∧
BMI mean (sd)	31.1 (7.4)	34.0 (8.2)#	33.4 (8.3)	34.0 (8.1)

Significant testing for comparisons between those with and without LEA. Categorical variables assessed with respect to trend. p-values

* <0.05

≤ 0.002

^∧ ≤ 0.0002

Table 2

Overall *NOS1AP* SNP frequency in a give cohort. MAF (minor allelic frequency) are presented for the CRIC study and for the appropriate HapMap studied populations release #28; CEU- Utah residents of European ancestry and YRI-Yoruban residents from Ibaden, Nigeria.

	White	**African-American**		
	rs1963645 (A>G)	**rs6659759 (T>C)**	**rs16849113 (C>T)**	**rs880296 (C>G)**
Wildtype	589 (38.0%)	611 (41.0%)	447 (30.0%)	560 (37.6%)
Heterozygote	729 (47.0%)	703 (47.2%)	758 (50.9%)	731 (49.1%)
Homozygote	232 (15.0%)	176 (11.8%)	285 (19.1%)	199 (13.4%)
MAF	0.385	0.354	0.446	0.379
MAF (HapMap)	0.333 (CEU)	0.394 (YRI)	0.483 (YRI)	0.391 (YRI)

Table 3

Association of *NOS1AP* with LEA or diabetes in the CRIC study. Odd ratios with 95% confidence intervals are presented. As described the full adjusted model includes gender, age, HbA1c, eGFR, PVD, BMI, and diabetes. In the final column the outcome was diabetes.

	Cohort	LEA	LEA (adjusted)	LEA among those with diabetes	Diabetes
rs1963645	White	1.73 (1.23, 2.44)	1.80 (1.21, 2.67)	1.90 (1.29, 2.81),	1.04 (0.90, 1.21)
rs6659759	African American	1.65 (1.21, 2.24)	1.64 (1.13, 2.39)	1.70 (1.21, 2.39)	0.96 (0.82,1.12)
rs16849113	African American	1.58 (1.16, 2.14)	1.60 (1.10, 2.32)	1.59 (1.14, 2.22)	1.01 (0.88, 1.18)
rs880296	African American	1.54 (1.14, 2.10)	1.77 (1.23,2.57)	1.49 (1.07, 2.09)	1.08 (0.93, 1.26)

Table 4

The number of individuals (percentage of those studied) at the UPENN CRIC site with loss of protective sensation (LOPS) as measured by Simmes-Weinstein filament. Presented based on a history of having diabetes or not having diabetes.

	CRIC		Diabetes		No Diabetes	
	Sample size	**LOPS**	**Sample Size**	**LOPS**	**Sample size**	**LOPS**
White	47	16(34%)	26	12(46.2%)	21	4(19.0%)
African American	50	26(52%)	30	18(60.0%)	20	8(40.0%)

Table 5

The odds ratio of association with 95% confidence intervals of our SNPs of interest with respect to loss of protective sensation (LOPS) in those with diabetes.

		Diabetes
rs1963645	White	16.97 (2.38, 120.97)
rs6659759	African American	3.02 (0.82, 11.12)
rs16849113	African American	3.62 (1.11, 11.83)
rs880296	African American	1.56 (0.51, 4.77)